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T cell epitopes in thrombopoietin

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T CELL EPITOPES IN THROMBOPOIETIN

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FIELD OF THE INVENTION

The present invention relates to polypeptides to be administered especially to humans and in particular for therapeutic use. The polypeptides are modified polypeptides whereby the modification results in a reduced propensity for the polypeptide to elicit an immune response upon administration to the human subject. The invention in particular relates to the modification of human thrombopoietin (TPO) to result in TPO proteins that are substantially non-immunogenic or less immunogenic than any non-modified counterpart when used *in vivo*. The invention relates furthermore to T-cell epitope peptides derived from said non-modified protein by means of which it is possible to create modified TPO variants with reduced immunogenicity.

BACKGROUND OF THE INVENTION

There are many instances where the efficacy of a therapeutic protein is limited by an unwanted immune reaction to the therapeutic protein. Several mouse monoclonal antibodies have shown promise as therapies in a number of human disease settings but in certain cases have failed due to the induction of significant degrees of a human anti-murine antibody (HAMA) response [Schroff, R. W. et al (1985) *Cancer Res.* 45: 879-885; Shawler, D.L. et al (1985) *J. Immunol.* 135: 1530-1535]. For monoclonal antibodies, a number of techniques have been developed in attempt to reduce the HAMA response [WO 89/09622; EP 0239400; EP 0438310; WO 91/06667]. These recombinant DNA approaches have generally reduced the mouse genetic information in the final antibody construct whilst increasing the human genetic information in the final construct. Notwithstanding, the resultant "humanized" antibodies have, in several cases, still elicited an immune response in patients [Issacs J.D. (1990) *Sem. Immunol.* 2: 449, 456; Rebello, P.R. et al (1999) *Transplantation* 68: 1417-1420].

Antibodies are not the only class of polypeptide molecule administered as a therapeutic agent against which an immune response may be mounted. Even proteins of human origin and with the same amino acid sequences as occur within humans can still induce an immune response in humans. Notable examples include the therapeutic use of granulocyte-macrophage colony stimulating factor [Wadhwa, M. et al (1999) *Clin. Cancer Res.* 5: 1353-1361] and interferon alpha 2 [Russo, D. et al (1996) *Br. J. Haem.*

94: 300-305; Stein, R. et al (1988) *New Engl. J. Med.* 318: 1409-1413]. In such situations where these human proteins are immunogenic, there is a presumed breakage of immunological tolerance that would otherwise have been operating in these subjects to these proteins.

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This situation is different where the human protein is being administered as a replacement therapy, for example in a genetic disease where there is a constitutional lack of the protein such as can be the case for diseases such as haemophilia A, Christmas disease, Gauchers disease and numerous other examples. In such cases, the therapeutic replacement protein may function immunologically as a foreign molecule from the outset, and where the individuals are able to mount an immune response to the therapeutic, the efficacy of the therapy is likely to be significantly compromised.

Irrespective of whether the protein therapeutic is seen by the host immune system as a foreign molecule, or if an existing tolerance to the molecule is overcome, the mechanism of immune reactivity to the protein is the same. Key to the induction of an immune response is the presence within the protein of peptides that can stimulate the activity of T-cells via presentation on MHC class II molecules, so-called "T-cell epitopes". Such T-cell epitopes are commonly defined as any amino acid residue sequence with the ability to bind to MHC class II molecules. Implicitly, a "T-cell epitope" means an epitope which when bound to MHC molecules can be recognised by a T-cell receptor (TCR), and which can, at least in principle, cause the activation of these T-cells by engaging a TCR to promote a T-cell response.

MHC Class II molecules are a group of highly polymorphic proteins which play a central role in helper T-cell selection and activation. The human leukocyte antigen group DR (HLA-DR) are the predominant isotype of this group of proteins however, isotypes HLA-DQ and HLA-DP perform similar functions. In the human population, individuals bear two to four DR alleles, two DQ and two DP alleles. There are approximately 70 different allotypes of the DR isotype, 30 different allotypes for DQ and 47 different allotypes for DP. The structure of a number of DR molecules has been solved and these appear as an open-ended peptide binding groove with a number of hydrophobic pockets which engage hydrophobic residues (pocket residues) of the peptide [Brown et al *Nature* (1993) 364: 33; Stern et al (1994) *Nature* 368: 215]. The MHC DR molecule is made of an alpha and

- a beta chain which insert at their C-termini through the cell membrane. Each heterodimer possesses a ligand binding domain which binds to peptides varying between 9 and 20 amino acids in length, although the binding groove can accommodate a maximum of 11 amino acids. DQ molecules have recently been shown to have an homologous structure and the DP family proteins are expected to be very similar. Polymorphism identifying the different allotypes of the class II molecule contributes to a wide diversity of different binding surfaces for peptides within the peptide binding groove and at the population level ensures maximal flexibility with regard to the ability to recognise foreign proteins and mount an immune response to pathogenic organisms.
- 5 An immune response to a therapeutic protein proceeds via the MHC class II peptide presentation pathway. Here exogenous proteins are engulfed and processed for presentation in association with MHC class II molecules of the DR, DQ or DP type. MHC Class II molecules are expressed by professional antigen presenting cells (APCs), such as macrophages and dendritic cells amongst others. Engagement of an MHC class II peptide complex by a cognate T-cell receptor on the surface of the T-cell, together with the cross-binding of certain other co-receptors such as the CD4 molecule, can induce an activated state within the T-cell. Activation leads to the release of cytokines further activating other lymphocytes such as B-cells to produce antibodies or activating T killer cells as a full cellular immune response.
- 10 T-cell epitope identification is the first step to epitope elimination, however there are few clear cases in the art where epitope identification and epitope removal are integrated into a single scheme. Thus WO98/52976 and WO00/34317 teach computational threading approaches to identifying polypeptide sequences with the potential to bind a sub-set of human MHC class II DR allotypes. In these teachings, predicted T-cell epitopes are removed by judicious amino acid substitution within the protein of interest. However with this scheme and other computationally based procedures for epitope identification [e.g. Godkin, A.J. et al (1998) *J. Immunol.* 161: 850-858; Sturniolo, T. et al (1999) *Nat. Biotechnol.* 17: 555-561], peptides predicted to be able to bind MHC class II molecules may not function as T-cell epitopes in all situations, particularly *in vivo* due to the effects of the processing pathways or other phenomena. In addition, the computational approaches to T-cell epitope prediction have in general not been capable of predicting epitopes with DP or DQ restriction although in general there is overlap in recognition between these systems.
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- Besides computational techniques, there are *in vitro* methods for measuring the ability of synthetic peptides to bind MHC class II molecules. An exemplary method uses B-cell lines of defined MHC allotype as a source of MHC class II binding surface and may be applied to MHC class II ligand identification [Marshall, K.W. et al (1994) *J. Immunol.* 152: 4946-4956; O'Sullivan et al (1990) *J. Immunol.* 145: 1799-1808; Robadey, C. et al (1997) *J. Immunol.* 159: 3238-3246]. However, such techniques are not adapted for the screening of multiple potential epitopes against a wide diversity of MHC allotypes, nor can they confirm the ability of a binding peptide to function as a T-cell epitope.
- 10 Recently techniques exploiting soluble complexes of recombinant MHC molecules in combination with synthetic peptides have come into use [Kern, F. et al (1998) *Nature Medicine* 4:975-978; Kwok, W.W. et al (2001) *TRENDS in Immunol.* 22:583-588]. These reagents and procedures are used to identify the presence of T-cell clones from peripheral blood samples from human or experimental animal subjects that are able to bind
- 15 particular MHC-peptide complexes. These procedures also are not readily adapted for the screening of multiple potential epitopes to a wide diversity of MHC allotypes.

- Biological assays of T-cell activation offer a practical option for providing a reading of the ability of a test peptide, or whole protein sequence, to evoke an immune response.
- 20 Examples of this kind of approach include the work of Petra et al using T-cell proliferation assays to the bacterial protein staphylokinase, followed by epitope mapping using synthetic peptides to stimulate T-cell lines [Petra. A.M. et al (2002) *J. Immunol.* 168: 155-161]. Similarly, T-cell proliferation assays using synthetic peptides of the tetanus toxin protein have resulted in definition of immunodominant regions of the toxin
- 25 [Reece, J.C. et al (1993) *J. Immunol.* 151: 6175-6184]. WO99/53038 discloses an approach whereby T-cell epitopes in a test protein may be determined using isolated sub-sets of human immune cells, promoting their differentiation *in vitro* and culture of the cells in the presence of synthetic peptides of interest and measurement of any induced proliferation in the cultured T-cells. The same technique is also described by Stickler et
- 30 al [Stickler, M.M. et al (2000) *J. Immunotherapy* 23: 654-660], where in both instances the method is applied to the detection of T-cell epitopes within bacterial subtilisin. Such a technique requires careful application of cell isolation techniques and cell culture with multiple cytokine supplements to obtain the desired immune cell sub-sets (dendritic cells,

CD4+ and or CD8+ T-cells) and is not conducive to rapid through-put screening using multiple donor samples.

As depicted above and as consequence thereof, it would be desirable to identify and to
5 remove or at least to reduce T-cell epitopes from a given in principal therapeutically
valuable but originally immunogenic peptide, polypeptide or protein. One of these
therapeutically valuable molecules is the human thrombopoietin molecule herein termed
"TPO".

TPO is a glycoprotein hormone involved regulation of platelet production. TPO promotes
10 both the proliferation of megakaryocyte progenitors in the bone marrow and their
maturation into platelet-producing megakaryocytes. The precursor form of the protein is
353 amino acid residues and cleavage of the N-terminal signal sequence produces a
mature protein of 332 amino acids. The mature protein comprises distinct regions with
the N-terminal domain highly conserved between mouse and man and significant
15 homology with erythropoietin and interferon-alpha and interferon-beta [de Sauvage, F.J.
et al (1994) *Nature* 369: 533-538; Chang, M. et al (1995) *J. Biol. Chem.* 270: 511-514].
The C-terminal domain has several sites for N-linked glycosylation. The N-terminal
domain is sufficient for the thrombopoietic effect of the molecule whereas the C-terminal
region is likely important in maintaining the circulating half-life *in vivo* [Foster, D. et al
20 (1996) *Stem Cells* 14: 102-107].

TPO has significant therapeutic value in the treatment of patients with reduced platelet
count. In particular patients with many types of cancer suffer thrombocytopenias on
account of myelosuppressive chemotherapy. Platelet transfusion has historically been the
mainstay by which such patients have been supported. The availability of purified TPO
25 from recombinant sources has enhanced the options available for aggressive
chemotherapy regimens and other patients at risk of bleeding complications due to their
thrombocytopenia [Prów, D. & Vadhan-Raj, S. (1998) *Oncology* 12: 1597-1608].

Despite the availability of therapeutic quantities of TPO, there is a continued need for
30 TPO analogues with enhanced properties. Desired enhancements include alternative
schemes and modalities for the expression and purification of the said therapeutic, but
also and especially, improvements in the biological properties of the protein. There is a
particular need for enhancement of the *in vivo* characteristics when administered to the
human subject. In this regard, it is highly desired to provide TPO with reduced or absent

potential to induce an immune response in the human subject. Others have provided TPO molecules and analogues [US, 5,989,538; US, 6,083,913; US, 5,879,673] including chemically modified and truncated forms [US, 5,989,538] and TPO fusion proteins [US, 6,066,318]. However none of these teachings recognise the importance of T cell epitopes to the immunogenic properties of the protein nor have been conceived to directly influence said properties in a specific and controlled way according to the scheme of the present invention.

It is a particular objective of the present invention to provide modified TPO proteins in which the immune characteristic is modified by means of reduced numbers of potential T-cell epitopes.

SUMMARY AND DESCRIPTION OF THE INVENTION

The present invention provides for modified forms of TPO, in which the immune characteristic is modified by means of reduced numbers of potential T-cell epitopes.

The invention discloses sequences identified within the TPO primary sequence that are potential T-cell epitopes by virtue of MHC class II binding potential.

The invention discloses also specific positions within the primary sequence of the molecule which according to the invention are to be altered by specific amino acid substitution, addition or deletion whilst retaining to a maximum degree the biological activity of the protein. In cases in which the loss of immunogenicity can be achieved only by a simultaneous loss of biological activity it is possible to restore the activity by further alterations within the amino acid sequence of the protein.

The invention furthermore discloses methods to produce such modified molecules, and above all methods to identify the T-cell epitopes which require alteration in order to reduce or remove immunogenic sites.

The present invention provides for modified forms of TPO proteins that are expected to display enhanced properties *in vivo*. The present invention discloses the major regions of the TPO primary sequence that are immunogenic in man and provides modification to the sequences to eliminate or reduce the immunogenic effectiveness of these sites.

In one embodiment, synthetic peptides comprising the immunogenic regions can be provided in pharmaceutical composition for the purpose of promoting a tolerogenic response to the whole molecule.

In a further embodiment, the modified TPO molecules of the present invention can be used in pharmaceutical compositions.

In summary the invention relates to the following issues:

- a modified molecule having the biological activity of TPO and being substantially non-immunogenic or less immunogenic than any non-modified molecule having the same biological activity when used *in vivo*;
- an accordingly specified molecule, wherein said loss of immunogenicity is achieved by removing one or more T-cell epitopes derived from the originally non-modified molecule;
- an accordingly specified molecule, wherein said loss of immunogenicity is achieved by reduction in numbers of MHC allotypes able to bind peptides derived from said molecule;
- an accordingly specified molecule, wherein one T-cell epitope is removed;
- an accordingly specified molecule, wherein said originally present T-cell epitopes are MHC class II ligands or peptide sequences which show the ability to stimulate or bind T-cells via presentation on class II;
- an accordingly specified molecule, wherein said peptide sequences are selected from the group of peptides a-g below wherein:
 - a) = LLVVMLLLTARLTLS,
 - b) = GEWKTQMEETKAQDI,
 - c) = QDILGAVTLLLEGVM,
 - d) = TLGAPDISSGTS DTG,
 - e) = SPTHPTGQYTLFPL,
 - f) = HPPTGQYTLFPLPPT,
 - g) = TGQYTLFPLPPTLPT,
- an accordingly specified molecule, wherein said peptide sequences are selected from the group of peptides as depicted in TABLE 1;
- an accordingly specified molecule, wherein said peptide sequences are selected from the group of peptides as depicted in TABLE 2;

- an accordingly specified molecule, wherein said peptide sequences are selected from the group of peptides as depicted in TABLE 3;
- an accordingly specified molecule, wherein 1 – 9 amino acid residues, preferably one amino acid residue in any of the originally present T-cell epitopes are altered;
- 5 • an accordingly specified molecule, wherein the alteration of the amino acid residues is substitution, addition or deletion of originally present amino acid(s) residue(s) by other amino acid residue(s) at specific position(s);
- an accordingly specified molecule, wherein, if necessary, additionally further alteration usually by substitution, addition or deletion of specific amino acid(s) is
10 conducted to restore biological activity of said molecule;
- a peptide molecule of above sharing greater than 90% amino acid identity with any of the peptide sequences a-g above;
- a peptide molecule of above sharing greater than 80% amino acid identity with any of the peptide sequences a-g above;
- 15 • a peptide molecule of above sharing greater than 90% amino acid identity with any of the peptide sequences of TABLE 1 or TABLE 2;
- a peptide molecule of above sharing greater than 80% amino acid identity with any of the peptide sequences of TABLE 1 or TABLE 2;
- peptide sequences as above able to bind MHC class II;
- 20 • an accordingly specified TPO molecule, wherein one or more of the amino acid substitutions is conducted at a position corresponding to any of the amino acids specified within any of peptides a-g above;
- an accordingly specified TPO molecule, wherein one or more of the amino acid substitutions is conducted at a position corresponding to any of the amino acids
25 specified within TABLE 1 or TABLE 2;
- a pharmaceutical composition comprising any of the peptides or modified peptides of above having the activity of binding to MHC class II
- a DNA sequence or molecule which codes for any of said specified modified molecules as defined above and below;
- 30 • a pharmaceutical composition comprising a modified molecule having the biological activity of TPO
- a pharmaceutical composition as defined above and / or in the claims, optionally together with a pharmaceutically acceptable carrier, diluent or excipient;

- a method for manufacturing a modified molecule having the biological activity of TPO as defined in any of the claims of the above-cited claims comprising the following steps: (i) determining the amino acid sequence of the polypeptide or part thereof; (ii) identifying one or more potential T-cell epitopes within the amino acid sequence of the protein by any method including determination of the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays; (iii) designing new sequence variants with one or more amino acids within the identified potential T-cell epitopes modified in such a way to substantially reduce or eliminate the activity of the T-cell epitope as determined by the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays; (iv) constructing such sequence variants by recombinant DNA techniques and testing said variants in order to identify one or more variants with desirable properties; and (v) optionally repeating steps (ii) – (iv);
- an accordingly specified method, wherein step (iii) is carried out by substitution, addition or deletion of 1 – 9 amino acid residues in any of the originally present T-cell epitopes;
- a 13mer T-cell epitope peptide having a potential MHC class II binding activity and created from non-modified TPO, selected from the group as depicted in TABLE 3 and its use for the manufacture of TPO having substantially no or less immunogenicity than any non-modified molecule with the same biological activity when used *in vivo*;
- a peptide sequence consisting of at least 9 consecutive amino acid residues of a 13mer T-cell epitope peptide as specified above and its use for the manufacture of TPO having substantially no or less immunogenicity than any non-modified molecule and having the biological activity of a human TPO when used *in vivo*;
- a 13mer T-cell epitope peptide having a potential MHC class II binding activity and created from non-modified TPO, selected from any of the group of sequences in TABLE 1 or TABLE 2 and its use for the manufacture of TPO having substantially no or less immunogenicity than any non-modified molecule and having the biological activity of human TPO when used *in vivo*;
- a peptide sequence consisting of at least 9 consecutive amino acid residues of a 13mer T-cell epitope peptide as derived from any of the sequences in TABLE 1 or TABLE 2 and its use for the manufacture of TPO having substantially no or less immunogenicity than any non-modified molecule and having the biological activity of human TPO when used *in vivo*.

The term "T-cell epitope" means according to the understanding of this invention an amino acid sequence which is able to bind MHC class II, able to stimulate T-cells and / or also to bind (without necessarily measurably activating) T-cells in complex with MHC class II.

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The term "peptide" as used herein and in the appended claims, is a compound that includes two or more amino acids. The amino acids are linked together by a peptide bond (defined herein below). There are 20 different naturally occurring amino acids involved in the biological production of peptides, and any number of them may be linked in any order to form a peptide chain or ring. The naturally occurring amino acids employed in the biological production of peptides all have the L-configuration. Synthetic peptides can be prepared employing conventional synthetic methods, utilizing L-amino acids, D-amino acids, or various combinations of amino acids of the two different configurations. Some peptides contain only a few amino acid units. Short peptides, e.g., having less than ten amino acid units, are sometimes referred to as "oligopeptides". Other peptides contain a large number of amino acid residues, e.g. up to 100 or more, and are referred to as "polypeptides". By convention, a "polypeptide" may be considered as any peptide chain containing three or more amino acids, whereas a "oligopeptide" is usually considered as a particular type of "short" polypeptide. Thus, as used herein, it is understood that any reference to a "polypeptide" also includes an oligopeptide. Further, any reference to a "peptide" includes polypeptides, oligopeptides, and proteins. Each different arrangement of amino acids forms different polypeptides or proteins. The number of polypeptides—and hence the number of different proteins—that can be formed is practically unlimited.

25 The invention may be applied to any TPO species of molecule with substantially the same primary amino acid sequences as those disclosed herein and would include therefore TPO molecules derived by genetic engineering means or other processes and may contain more or less than 332 amino acid residues.

30 According to the methods described herein, the inventors have discovered the regions of the TPO molecule comprising the critical T-cell epitopes driving the immune responses to this protein.

The general method of the present invention leading to the modified TPO comprises the following steps:

- (a) determining the amino acid sequence of the polypeptide or part thereof;
- (b) identifying one or more potential T-cell epitopes within the amino acid sequence of the protein by any method including determination of the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays;
- (c) designing new sequence variants with one or more amino acids within the identified potential T-cell epitopes modified in such a way to substantially reduce or eliminate the activity of the T-cell epitope as determined by the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays. Such sequence variants are created in such a way to avoid creation of new potential T-cell epitopes by the sequence variations unless such new potential T-cell epitopes are, in turn, modified in such a way to substantially reduce or eliminate the activity of the T-cell epitope; and
- (d) constructing such sequence variants by recombinant DNA techniques and testing said variants in order to identify one or more variants with desirable properties according to well known recombinant techniques.

The identification of potential T-cell epitopes according to step (b) can be carried out according to methods described previously in the art. Suitable methods are disclosed in WO 98/59244; WO 98/52976; WO 00/34317; WO 02/069232 and may be used to identify binding propensity of TPO-derived peptides to an MHC class II molecule. In practice, the compositions embodied in the present invention have been derived with the concerted application of biological *ex vivo* human T-cell proliferation assays and a software tool exploiting the scheme outlined in WO 02/069232 and which is an embodiment of the present invention.

The software simulates the process of antigen presentation at the level of the peptide MHC class II binding interaction to provide a binding score for any given peptide sequence. Such a score is determined for many of the predominant MHC class II allotypes extant in the population. As this scheme is able to test any peptide sequence, the consequences of amino acid substitutions additions or deletions with respect to the ability of a peptide to interact with a MHC class II binding groove can be predicted. Consequently new sequence compositions can be designed which contain reduced numbers of peptides able to interact with the MHC class II and thereby function as

immunogenic T-cell epitopes. Where the biological assay using any one given donor sample can assess binding to a maximum of 4 DR allotypes, the *in silico* process can test the same peptide sequence using >40 allotypes simultaneously. In practice this approach is able to direct the design of new sequence variants which are compromised in the their
5 ability to interact with multiple MHC allotypes.

By way of an example of this *in silico* approach, the results of an analysis conducted on the human TPO sequence is provided as TABLE 3. Therein are listed 13mer peptide sequences derived from TPO detected to have the capability to bind one or more MHC
10 class II allotypes with a significant binding score. Taken in its entirety, this dataset of 13mer peptides is considered to provide with a high degree of certainty, the universe of permissible MHC class ligands for the human TPO protein. For reasons such as the requirement for proteolytic processing of the complete TPO polypeptide and other physiologic steps leading to the presentation of TPO peptides *in vivo*, it would be clear
15 that a relatively minor sub-set of the entire repertoire of peptides will have ultimate biological relevance. In order to further identify such biologically relevant peptides, the inventors have developed an approach exploiting *ex vivo* human T-cell proliferation assays.

20 This approach has proven to be a particularly effective method and is disclosed herein as an embodiment of the invention. The method can be applied to test part of the sequence, for example selected regions of the sequence such as a sub-set of TPO peptides such as all or some of those listed in TABLE 3; or the method may be applied to test whole TPO sequence. In the present studies, the method has involved the testing of overlapping
25 TPO-derived peptide sequences in a scheme so as to scan and test the full TPO sequence of 161 residues. The synthetic peptides are tested for their ability to evoke a proliferative response in human T-cell cultured *in vitro*. Where this type of approach is conducted using naïve human T-cells taken from healthy donors, the inventors have established that in the operation of such an assay, a stimulation index equal to or greater than 2.0 is a
30 useful measure of induced proliferation. The stimulation index is conventionally derived by division of the proliferation score (e.g. counts per minute of radioactivity if using ³H-thymidine incorporation) measured to the test peptide by the score measured in cells not contacted with a test peptide.

The present studies have uncovered 27 peptide sequences able to evoke a significant proliferative response (i.e. SI>2.0). These peptides are listed in TABLE 1 and are an embodiment of the invention. Within this set of peptides, a further sub-set of 7 peptides have been identified which evoke a significant proliferative response in 2 or more individual donor samples and for some of these responses the magnitude of response has indeed been significantly higher than SI=2.0. These peptides are listed in TABLE 2 and are a further embodiment of the invention.

The disclosed peptide sequences herein represent the critical information required for the construction of modified TPO molecules in which one or more of these epitopes is compromised. Under the scheme of the present, the epitopes are compromised by mutation to result in sequences no longer able to function as T-cell epitopes. It is possible to use recombinant DNA methods to achieve directed mutagenesis of the target sequences and many such techniques are available and well known in the art.

Where it is the objective of this invention to modify the amino acid sequences of at least one or more of the above listed peptides from TABLE 1, it is most preferred to modify the sequence of one or more of the peptides P3, P24, P28, P90, P99, P100 and P101 as identified in TABLE 2. From the foregoing it can be seen that according to this invention a number of variant TPO proteins can be produced and tested for the desired immune and functional characteristic

TABLE 1:

TPO peptide sequences able to stimulate ex-vivo human T-cells.

Peptide No	TPO; 15mer peptide sequence	Residue#
1	MELTELLLVMLLLT	1
2	TELLLVMLLLTARL	4
3	LLVMLLLTARLTLS	7
6	ARLTLSPPAPACDL	16
16	RLSQCEVHPLPTFV	46
24	GEWKTQMEETKAQDI	70
28	QDILGAVTLLLEGVM	82
31	LLEGVMAARGQLGPT	91

35	GPTCLSSLLGQLSGQ	103
38	GQLSGQVRLLLGALQ	112
39	SGQVRLLLGALQSL	115
47	TTAHKDPNAIFLSFQ	139
53	RGKVRFLMLVGGSTL	157
60	PTTAVPSRTSLVLT	178
70	TASARTTGSGLLKWQ	208
75	QGFRAKIPGLLNQTS	223
81	DQIPGYLNRIHELLN	241
84	IHELLNGTRGLFPGP	250
90	TLGAPDISSGTS DTG	267
97	QPGYSPSPTHPPTGQ	288
99	SPTHPTTGQYTLFPL	294
100	HPPTGQYTLFPLPPT	297
101	TGQYTLFPLPPTLPT	300
105	LPTTPWQLHPLLPDP	312
106	TPWQLHPLLPDPSAP	315
113	PLLNTSYTHSQNLSQ	336
114	NTSYTHSQNLSQEGA	339

TABLE 2:

TPO peptide sequences able to stimulate ex vivo human T-cells from 2 or more donor samples

Peptide No	TPO; 15mer peptide sequence	Residue#
P3	LLVVMLLLTARLTLS	7
P24	GEWKTQMEETKAQDI	70
P28	QDILGAVTLLLEGVM	82
P90	TLGAPDISSGTS DTG	267
P99	SPTHPTTGQYTLFPL	294
P100	HPPTGQYTLFPLPPT	297
P101	TGQYTLFPLPPTLPT	300

5

The variant proteins will most preferably be produced by recombinant DNA techniques although other procedures including chemical synthesis of TPO fragments may be contemplated. It is a facile procedure to use the protein sequence information provided

herein to deduce polynucleotides (DNA) encoding any of the preferred variant TPO molecules or fragments. This is most readily achieved using computer software tools such as the DNASTar software suite [DNASTar Inc. Madison WI, USA] or similar. Any such DNA sequence encoding the polypeptides of the present or significant homologues thereof should be considered as embodiments of this invention.

The invention relates to TPO analogues in which substitutions of at least one amino acid residue have been made at positions resulting in a substantial reduction in activity of or elimination of one or more potential T-cell epitopes from the protein. One or more amino acid substitutions at particular points within any of the potential MHC class II ligands identified in TABLE 3 or more preferably peptide epitope sequences of TABLES 1 and 2 may result in a TPO molecule with a reduced immunogenic potential when administered as a therapeutic to the human host.

15 TABLE 3

Peptide sequences in human TPO with potential human MHC class II binding activity.

	APPACDLRVLSKL,	ACDLRVLSKLLRD,	LRVLSKLLRDShV,
	RVLSKLLRDShVL,	VLSKLLRDShVLH,	SKLLRDShVLHSR,
	KLLRDShVLHSRL,	LRDShVLHSRLSQ,	RDShVLHSRLSQC,
20	SHVLHSRLSQCPE,	HVLHSRLSQCPEV,	SRLSQCPEVHPLP,
	PEVHPLPTPVLLP,	EVHPLPTPVLLPA,	HPLPTPVLLPAVD,
	PLPTPVLLPAVDF,	PTPVLLPAVDFSL,	TPVLLPAVDFSLG,
	PVLLPAVDFSLGE,	VLLPAVDFSLGEW,	PAVDFSLGEWKTQ,
	VDFSLGEWKTQME,	FSLGEWKTQMEET,	GEWKTQMEETKAQ,
25	EWKTQMEETKAQD,	TQMEETKAQDILG,	EETKAQDILGAVT,
	KAQDILGAVTLLL,	QDILGAVTLLLEG,	DILGAVTLLLEGV,
	LGAVTLLLEGVMA,	GAVTLLLEGVMAA,	VTLLLEGVMAARG,
	TLLLEGVMAARGQ,	LLLEGVMAARGQL,	LEGVMAARGQLGP,
	EGVMAARGQLGPT,	GVMAARGQLGPTC,	AARGQLGPTCLSS,
30	GQLGPTCLSSLLG,	TCLSSLLGQLSGQ,	SSLLGQLSGQVRL,
	SLLGQLSGQVRLL,	LLGQLSGQVRLLL,	GQLSGQVRLLLGA,
	GQVRLLLGALQSL,	VRLLLGALQSLLG,	RLLLGALQSLLGT,
	LLLALQSLLGTQ,	GALQSLLGTQLPP,	ALQSLLGTQLPPQ,
	QSLLGTQLPPQGR,	SLLGTQLPPQGRT,	TQLPPQGRTTAHK,
35	TTAHKDPNAIFLS,	PNAIFLSFQHLLR,	NAIFLSFQHLLRG,
	AIFLSFQHLLRGK,	IFLSFQHLLRGKV,	LSFQHLLRGKVRF,

	FQHLLRGKVRFLM,	QHLLRGKVRFLML,	HLLRGKVRFLMLV,
	GKVRFLMLVGGST,	VRFLMLVGGSTLC,	RFLMLVGGSTLCV,
	FLMLVGGSTLCVR,	LMLVGGSTLCVRR	MLVGGSTLCVRR,
	STLCVRRAPPTTA,	LCVRRAPPTTAVP,	TAVPSRTSLVLT,
5	RTSLVLTNLNLPN,	TSLVLTNLNLPNR,	SLVLTNLNLPNRT,
	LVLTLNLPNRTS,	LTLNLPNRTSGL,	NLPNRTSGLLET,
	NRTSGLLETNFTA,	RTSGLLETNFTAS,	SGLLETNFTASAR,
	GLLETNFTASART,	TNFTASARTTGSG,	GSGLLKWQQGFRA,
	SGLLKWQQGFRAK,	GLLKWQQGFRAKI,	LKWQQGFRAKIPG,
10	QGFRAKIPGLLNQ,	AKIPGLLNQTSRS,	PGLLNQTSRSLDQ,
	GLLNQTSRSLDQI,	TSRSLDQIPGYLN,	SRSLDQIPGYLNR,
	RSLDQIPGYLNRI,	DQIPGYLNRIHEL,	PGYLNRIHELLNG,
	GYLNRIHELLNGT,	NRIHELLNGTRGL,	IHELLNGTRGLFP,
	HELLNGTRGLFPG,	ELLNGTRGLFPGP,	RGLFPGPSRRTLGA,
15	GLFPGPSRRTLGA,	PSRRTLGAAPDISS,	RTLGAAPDISSGTS,
	PDISSGTSDTGSL,	SDTGSLPPNLQPG,	GSLPPNLQPGYSP,
	PNLQPGYSPSPTH,	PGYSPSPTHPPTG,	PTHPTGQYTLFP,
	GQYTLFPPLPPTLP,	YTLFPPLPPTLPTP,	TLFPPLPPTLPTPV,
	FPLPPTLPTPVVQ,	PLPPTLPTPVVQL,	PTLPTPVVQLHPL,
20	TPVVQLHPLLPDP,	PVVQLHPLLPDPS,	VQLHPLLPDPSAP,
	HPLLPDPSAPTPT,	PLLPDPSAPTPTP,	SPLLNNTSYTHSQN,
	PLLNNTSYTHSQNL,	TSYTHSQNLQEG	

It is most preferred to provide an TPO molecule in which amino acid modification (e.g. a substitution) is conducted within the most immunogenic regions of the parent molecule. For the elimination of T-cell epitopes, amino acid substitutions are preferably made at appropriate points within the peptide sequence predicted to achieve substantial reduction or elimination of the activity of the T-cell epitope. In practice an appropriate point will preferably equate to an amino acid residue binding within one of the pockets provided within the MHC class II binding groove. It is most preferred to alter binding within the first pocket of the cleft at the so-called P1 or P1 anchor position of the peptide. The quality of binding interaction between the P1 anchor residue of the peptide and the first pocket of the MHC class II binding groove is recognised as being a major determinant of overall binding affinity for the whole peptide. An appropriate substitution at this position of the peptide will be for a residue less readily accommodated within the pocket, for example, substitution to a more hydrophilic residue. Amino acid residues in the peptide

at positions equating to binding within other pocket regions within the MHC binding cleft are also considered and fall under the scope of the present invention.

As will be clear to the person skilled in the art, multiple alternative sets of substitutions
5 could be arrived at which achieve the objective of removing un-desired epitopes. The resulting sequences would however be recognised to be closely homologous with the specific compositions disclosed herein and therefore fall under the scope of the present invention.

10 It is understood that single amino acid substitutions within a given potential T-cell epitope are the most preferred route by which the epitope may be eliminated. Combinations of substitution within a single epitope may be contemplated and for example can be particularly appropriate where individually defined epitopes are in overlap with each other. Moreover, amino acid substitutions either singly within a given epitope or in
15 combination within a single epitope may be made at positions not equating to the "pocket residues" with respect to the MHC class II binding groove, but at any point within the peptide sequence. Substitutions may be made with reference to an homologues structure or structural method produced using *in silico* techniques known in the art and may be based on known structural features of the molecule according to this invention. All such
20 substitutions fall within the scope of the present invention.

Amino acid substitutions other than within the peptides identified above may be contemplated particularly when made in combination with substitution(s) made within a listed peptide. For example a change may be contemplated to restore structure or
25 biological activity of the variant molecule. Such compensatory changes and changes to include deletion or addition of particular amino acid residues from the TPO polypeptide resulting in a variant with desired activity and in combination with changes in any of the disclosed peptides fall under the scope of the present.

30 In as far as this invention relates to modified TPO, compositions containing such modified TPO proteins or fragments of modified TPO proteins and related compositions should be considered within the scope of the invention. A pertinent example in this respect could be development of peptide mediated tolerance induction strategies wherein one or more of the disclosed peptides is administered to a patient with immunotherapeutic

intent. Accordingly, synthetic peptides molecules, for example one of more of those listed in TABLE 1, are considered embodiments of the invention. In another aspect, the present invention relates to nucleic acids encoding modified TPO entities. In a further aspect the present invention relates to methods for therapeutic treatment of humans using the modified TPO proteins. In a further aspect still, the invention relates to methods for therapeutic treatment using pharmaceutical preparations comprising peptide or derivative molecules with sequence identity or part identity with the sequences herein disclosed.

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PATENT CLAIMS

1. A modified human thrombopoietin (TPO) molecule being substantially non-immunogenic or less immunogenic than non-modified human TPO and having
5 essentially the same biological specificity and activity when used in vivo, comprising specifically altered amino acid residues compared with the non-modified parental molecule, wherein said altered amino acid residues cause a reduction or an elimination of one or more of T-cell epitopes which act in the parental non-modified molecule as MHC class II binding ligands and stimulate T-cells.
10
2. A modified TPO molecule according to claim 1, wherein said alterations are made at one or more positions within one or more of the strings of contiguous amino acid residues present in the parental molecule as depicted in Table 1.
15
3. A modified TPO molecule according to claim 2, wherein said alterations are made at one or more positions within one or more of the strings of contiguous amino acid residues present in the parental molecule as depicted in Table 2.
- 20 4. A modified TPO molecule according to claim 1, wherein said alterations are made at one or more positions within one or more of the strings of contiguous amino acid residues present in the parental molecule as depicted in Table 3.
- 25 5. A modified TPO molecule according to any of the claims 1 to 4, wherein said alterations are substitutions of 1 - 9 amino acid residues.
- 30 6. A modified TPO molecule according to any of the claims 1 to 5, wherein when tested as a whole protein in a biological assay of induced cellular proliferation of human T-cells exhibits a stimulation index (SI) smaller than the parental molecule and smaller than 2 tested in parallel using cells from the same donor wherein said index is taken as the value of cellular proliferation scored following stimulation by the protein and divided by the value of cellular proliferation scored in control cells not in receipt of protein and wherein cellular proliferation is measured by any suitable means.

7. A DNA sequence coding for a TPO molecule as defined in any of the claims 1 to 6.
8. A pharmaceutical composition comprising a modified TPO molecule of any of the preceding claims, optionally together with a pharmaceutically acceptable carrier, diluent or excipient.
9. A peptide molecule selected from Table 1, 2 or 3 having a potential MHC class II binding activity and created from the primary sequence of non-modified human TPO, whereby said peptide molecule has a stimulation index of > 1.8 in a biological assay of cellular proliferation, wherein said index is taken as the value of cellular proliferation scored following stimulation by a peptide and divided by the value of cellular proliferation scored in control cells not in receipt peptide and wherein cellular proliferation is measured by any suitable means.
- 10: A modified peptide molecule deriving from the peptide molecule of claim 9 by amino acid substitution, having a reduced or absent potential MHC class II binding activity expressed by a stimulation index of less than 2, whereby said index is taken as the value of cellular proliferation scored following stimulation by a peptide and divided by the value of cellular proliferation scored in control cells not in receipt peptide and wherein cellular proliferation is measured by any suitable means.
11. A DNA sequence coding for a peptide of claim 9 or 10.
12. Use of a modified peptide according to claim 10 for the manufacture of a modified TPO molecule as defined in claim 1.
13. Use of a peptide according to claim 9 for the manufacture of a vaccine in order to reduce immunogenicity to TPO in a patient.

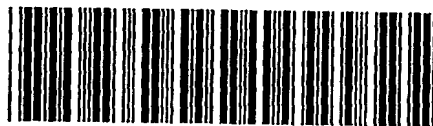
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ABSTRACT

The invention relates to the modification of human thrombopoietin (TPO) to result in TPO proteins that are substantially non-immunogenic or less immunogenic than any non-
5 modified counterpart when used *in vivo*. The invention relates furthermore to T-cell epitope peptides derived from said non-modified protein by means of which it is possible to create modified TPO variants with reduced immunogenicity.

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